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## **Thermal conditioning improves quality and speed of keratinocyte sheet production for burn wound treatment**

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**Abstract:** **BACKGROUND** AIMS Cultured patient-specific keratinocyte sheets have been used clinically since the 1970s for the treatment of large severe burns. However, despite significant developments in recent years, successful and sustainable treatment is still a challenge. Reliable, high-quality grafts with faster availability and a flexible time window for transplantation are required to improve clinical outcomes. **METHODS** Keratinocytes are usually grown in vitro at 37°C. Given the large temperature differences in native skin tissue, the aim of the authors' study was to investigate thermal conditioning of keratinocyte sheet production. Therefore, the influence of 31°C, 33°C and 37°C on cell expansion and differentiation in terms of proliferation and sheet formation efficacy was investigated. In addition, the thermal effect on the biological status and thus the quality of the graft was assessed on the basis of the release of wound healing-related biofactors in various stages of graft development. **RESULTS** The authors demonstrated that temperature is a decisive factor in the production of human keratinocyte sheets. By using specific temperature ranges, the authors have succeeded in optimizing the individual manufacturing steps. During the cell expansion phase, cultivation at 37°C was most effective. After 6 days of culture at 37°C, three times and six times higher numbers of viable cells were obtained compared with 33°C and 31°C. During the cell differentiation and sheet formation phase, however, the cells benefited from a mildly hypothermic temperature of 33°C. Keratinocytes showed increased differentiation potential and formed better epidermal structures, which led to faster biomechanical sheet stability at day 18. In addition, a cultivation temperature of 33°C resulted in a longer lasting and higher secretion of the investigated immunomodulatory, anti-inflammatory, angiogenic and pro-inflammatory biofactors. **CONCLUSIONS** These results show that by using specific temperature ranges, it is possible to accelerate the large-scale production of cultivated keratinocyte sheets while at the same time improving quality. Cultivated keratinocyte sheets are available as early as 18 days post-biopsy and at any time for 7 days thereafter, which increases the flexibility of the process for surgeons and patients alike. These findings will help to provide better clinical outcomes, with an increased take rate in severe burn patients.

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## FULL-LENGTH ARTICLE

## Manufacturing

# Thermal conditioning improves quality and speed of keratinocyte sheet production for burn wound treatment



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## ABSTRACT

**Background aims:** Cultured patient-specific keratinocyte sheets have been used clinically since the 1970s for the treatment of large severe burns. However, despite significant developments in recent years, successful and sustainable treatment is still a challenge. Reliable, high-quality grafts with faster availability and a flexible time window for transplantation are required to improve clinical outcomes.

**Methods:** Keratinocytes are usually grown *in vitro* at 37°C. Given the large temperature differences in native skin tissue, the aim of the authors' study was to investigate thermal conditioning of keratinocyte sheet production. Therefore, the influence of 31°C, 33°C and 37°C on cell expansion and differentiation in terms of proliferation and sheet formation efficacy was investigated. In addition, the thermal effect on the biological status and thus the quality of the graft was assessed on the basis of the release of wound healing-related biofactors in various stages of graft development.

**Results:** The authors demonstrated that temperature is a decisive factor in the production of human keratinocyte sheets. By using specific temperature ranges, the authors have succeeded in optimizing the individual manufacturing steps. During the cell expansion phase, cultivation at 37°C was most effective. After 6 days of culture at 37°C, three times and six times higher numbers of viable cells were obtained compared with 33°C and 31°C. During the cell differentiation and sheet formation phase, however, the cells benefited from a mildly hypothermic temperature of 33°C. Keratinocytes showed increased differentiation potential and formed better epidermal structures, which led to faster biomechanical sheet stability at day 18. In addition, a cultivation temperature of 33°C resulted in a longer lasting and higher secretion of the investigated immunomodulatory, anti-inflammatory, angiogenic and pro-inflammatory biofactors.

**Conclusions:** These results show that by using specific temperature ranges, it is possible to accelerate the large-scale production of cultivated keratinocyte sheets while at the same time improving quality. Cultivated keratinocyte sheets are available as early as 18 days post-biopsy and at any time for 7 days thereafter, which increases the flexibility of the process for surgeons and patients alike. These findings will help to provide better clinical outcomes, with an increased take rate in severe burn patients.

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## Introduction

Skin provides an invaluable protective barrier for the human body. If the skin or other tissues are injured by heat, cold, electricity, chemicals, friction or radiation, it is referred to as a burn. Burns are a serious public health problem worldwide. According to the World Health Organization, around 180 000 people die of burns every year [1]. After a severe burn of over 40% of the total body surface,

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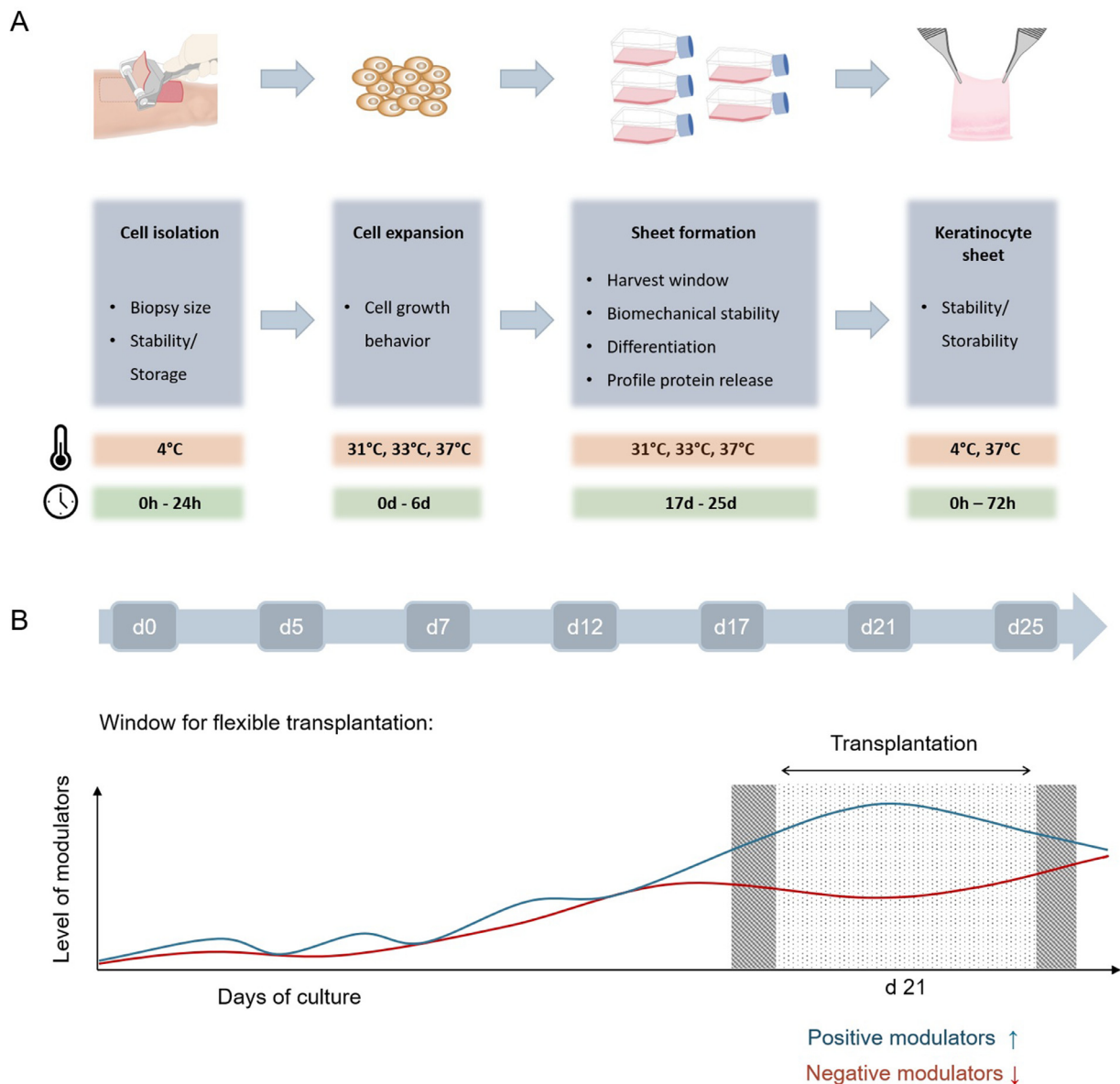
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patients suffer from dramatic loss of their epidermal and dermal layers, and the natural repair mechanisms are also significantly damaged. These serious injuries lead to a state of immunosuppression, which predisposes burn patients to infectious complications. Patients are at high risk of infection from inhalation injuries and for the development of sepsis secondary to pneumonia, catheter-related infections or suppurative thrombophlebitis [2]. In these patients, the remaining unaffected skin areas are often not sufficient to provide autograft coverage.

To treat this extensive skin damage, large-scale *in vitro* cultivated skin grafts can be made from the patient's own cells (autologous). Keratinocytes, the main cell type present in the epidermis, are isolated from an autologous skin biopsy and cultivated *in vitro* to form a multi-layer cell sheet (Figure 1). These keratinocyte sheets can be transferred to carriers (e.g., Vaseline gauzes) to facilitate subsequent implantation [3]. The original technique was developed by Rheinwald and Green [4] and Green *et al.* [5] in the 1970s. However, despite considerable progress in the large-scale production of keratinocyte sheets in recent years, their

successful grafting remains challenging. In fact, graft take rates show enormous variability, especially in deep and infected burn wounds, which necessitates further improvements [6]. For example, a highly inflammatory situation in combination with low neovascularization can be problematic for successful grafting. The use of cultivated skin grafts represents a possibility for the treatment of extensive burn lesions since they allow the resurfacing of a significant area of burn that cannot be achieved with other surgical techniques based on autologous grafts [7]. Compared with allografts, xenografts, dermal substitutes and biosynthetic dressings, however, the production of cultured skin grafts takes a long time, up to 3–4 weeks [8,9]. During this period, the patient's state of health cannot be predicted. To be able to react more flexibly to unforeseen clinical incidents in the patient, an extended and mainly shorter time frame for transplantation would be helpful. For this reason, the authors attempted modifications to the large-scale keratinocyte sheet manufacturing protocol to create an extended flexible transplantation window that allows the surgeon to adjust the time of transplantation according to the patient's state of health.



**Fig. 1.** (A) Schematic representation of autologous keratinocyte sheet production. After skin biopsy, the epidermal cells are isolated and expanded. After reaching the required number of cells, sheet formation is initiated. Thereafter, the keratinocyte sheet can be harvested and transplanted. For production optimization, the effect of temperature and time on the different production steps of cell isolation, cell expansion and sheet formation during keratinocyte sheet production was investigated. (B) To find a possible time window for flexible transplantation, the keratinocyte sheets were examined with regard to their positive (blue) and negative (red) modulators between day 17–25 of total culture. (Color version of figure is available online).

The native skin and its constituent cells, such as keratinocytes, are exposed to large temperature differences. The cells have to go through a temperature gradient from 37°C in the innermost basal layer to 32°C on the skin surface [10]. Since temperature appears to be involved in several pathways that regulate the balance between proliferation and differentiation of human keratinocytes, optimized temperature conditions could be used in a targeted manner to accelerate the process of keratinocyte sheet production and outcome optimization.

The effect of hypothermic culture on large-scale keratinocyte sheet manufacturing and engraftment properties has not yet been described. The authors hypothesized that temperature—specifically hypothermic conditions—may alter and perhaps even enhance the production of wound healing-related biofactors. This would improve overall keratinocyte sheet adhesion to the wound bed and, by extension, provide better clinical results, with an enhanced graft take rate in patients.

To give future burn patients the best possible treatment, the aim of the authors' study was to (i) expand the implantability window and speed up production (time) and (ii) characterize and maximize the activity of implanted keratinocyte sheets (quality). The influence of temperature on the individual *in vitro* phases of keratinocyte sheet manufacturing, such as biopsy storage, cell isolation, cell expansion and sheet formation, was investigated (Figure 1). The main focus was on the reliability and quality of the keratinocyte sheets. In addition to biomechanical stability (sheet thickness), the latter also includes the biological status of the sheets, such as cell differentiation status, biological properties and activity. With regard to a transplantation, the biological properties of cultivated skin grafts have to be ideal for the harsh environment of a debrided burn to successfully reconstitute the skin tissue. Dynamic levels of growth factors, anti- and pro-inflammatory cytokines and other important wound healing modulators secreted by keratinocyte sheets were evaluated at various stages of their *in vitro* development to determine the quality of the sheet cultures.

## Methods

### Source of human keratinocytes

For the present study, human keratinocytes were isolated from split- or full-thickness skin samples collected from female patients undergoing plastic surgery at the Division of Plastic Surgery and Hand Surgery of the University Hospital Zürich, Switzerland. The informed consent was obtained for experimentation with human material and the privacy rights of the patients participating in the study were always be observed (institutional ethics committee license KEK-ZH-2014-0197). The skin tissue samples were retrieved from remnant operative tissue, which would otherwise have been discarded, excised from the breast or abdominal region. Patient age range and sample number are specified for each assay in the following sections.

### Isolation of human keratinocytes

The skin biopsies were placed in a Petri dish and the surface area determined with a ruler. The isolation of keratinocytes from full- or split-thickness samples was performed using the same procedure. Briefly, the skin tissue was micro-dissected in small pieces of approximately 10 × 10 mm and digested with Dispase solution (Corning, New York, USA) 12 U/mL in phosphate-buffered saline (PBS) for 15–18 h at 4°C. A maximum biopsy size of 8 cm<sup>2</sup> was digested in 15 mL Dispase to maintain enzyme efficiency. Thereafter, the epidermis and dermis were mechanically separated. For isolation of the epidermal cell fraction, the epidermal layer was further digested with trypsin/ethylenediaminetetraacetic acid 5x (Thermo Fisher Scientific, Waltham, MA, USA) for 15–20 min at 37°C. The digestion was halted by adding 3.75 mg/mL soybean trypsin inhibitor (Thermo Fisher

Scientific). The cell isolation efficiency was evaluated in 59 biopsy samples (patient age range, 42.2 ± 12.4 years).

To that end, a sample of the isolated cells from each biopsy was collected and stained with Acridine Orange and 4',6'-diamidino-2-phenylindole to determine the number of isolated cells as well as their viability (NucleoCounter NC-200; ChemoMetec, Lillerød, Denmark). The isolation efficiency was reported per biopsy sample as isolated viable cells/cm<sup>2</sup> biopsy. In addition, the authors examined whether the age of the donors had a significant impact on the isolation efficiency. The mean ± standard deviation (SD) of the isolation efficiency was calculated. Furthermore, the Pearson correlation was evaluated between isolated viable cells and biopsy surface area as well as between isolated viable cells/cm<sup>2</sup> biopsy and donor age. An independent two-sided *t*-test was performed to compare the number of viable cells/cm<sup>2</sup> biopsy isolated after a 6-h versus 24-h storage period. *P* < 0.05 indicated statistical significance.

### Biopsy storability

To test the effect of cold temperature storage on biopsy viability, four biopsies (patient age range, 44.5 ± 1.6 years) were divided into five equal pieces each and placed in a closed and sterile container at 4°C in maintenance medium CnT-XP3 (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) in a refrigerator. One piece of each biopsy was taken out of storage after 0, 6, 12, 18 and 24 h. Cells were isolated and assessed for the number of isolated viable cells using the NucleoCounter. The number of isolated viable cells/cm<sup>2</sup> biopsy from all four biopsy samples was reported for each time point.

### Human keratinocyte proliferation

To analyze the effect of thermal conditioning on cell proliferation kinetics, keratinocytes from three patient biopsies (patient age range, 47.3 ± 24.5 years) were seeded at 10 000 cells/cm<sup>2</sup> in 24-well plates coated with bovine collagen I 6 µg/cm<sup>2</sup> (Symatase, Chaponost, France). Cells were cultivated at 37°C, 33°C and 31°C in triplicate using the chemically defined and xenogeneic-free expansion medium CnT-Prime (CELLnTEC Advanced Cell Systems AG) at 37 °C in a humidified atmosphere and 5% carbon dioxide (CO<sub>2</sub>). To minimize the risk of contamination, the medium was supplemented with 100 U/mL penicillin (Sigma-Aldrich, St Louis, MO, USA) and 100 µg/mL streptomycin (Sigma-Aldrich) for the first 3 days of culture. Every 2–3 days, the culture medium was changed with unsupplemented CnT-Prime medium until cells reached 70–90% confluency.

A colorimetric proliferation assay with Crystal Violet (Sigma-Aldrich) was performed to determine the number of viable cells in the monolayer cultures during the expansion phase after 0, 24, 72 and 144 h. A standard curve was established by seeding keratinocytes at 100 000, 50 000, 25 000, 12 500, 6250 and 0 cells/well in a 24-well plate and measuring the Crystal Violet assay signal at day 0. At the individual time points, the cells were fixed with methanol and stained with 0.1% Crystal Violet for 5 min. The cell layer was dried and decolorized with a sodium dodecyl sulfate solution (0.5% with ethanol 50% in 0.5 M tris[hydroxymethyl]aminomethane hydrochloride, pH 7.8) for 60 min at 37°C. The optical density of the supernatant was read at the absorption maximum of 590 nm. Using the internal standard, the mean ± SD of viable cells/cm<sup>2</sup> across all three biopsies was calculated for each time point. A one-way analysis of variance with a Bonferroni post-hoc test was performed for each time point, comparing the three temperatures. *P* < 0.05 was chosen as the statistically significant threshold.

### Keratinocyte sheet formation

To analyze the influence of temperature on the production and quality of keratinocyte sheets, differentiation of keratinocytes and sheet



formation at different temperatures were analyzed. Freshly isolated keratinocytes of six patient biopsies (patient age range,  $46.0 \pm 8.1$  years) were seeded at a density of 30 000 viable cells/cm<sup>2</sup> on flasks coated with bovine collagen I and expanded at 37°C in a humidified atmosphere and 5% CO<sub>2</sub>. After reaching confluency, production of keratinocyte sheets was initiated by passaging the cells and plating them at a density of 5000 viable cells/cm<sup>2</sup> in CnT-Prime on T115 peel-off flasks (Techno Plastic Products AG, Trasadingen, Switzerland) coated with bovine collagen I. When the cells reached 80–90% confluency after 3–5 days, keratinocyte differentiation was induced using the chemically defined and xenogeneic-free differentiation medium CnT-Prime 2D (CELLnTEC Advanced Cell Systems AG) supplemented with 1.2 mM calcium chloride (Sigma-Aldrich). Henceforth, the culture flasks were cultivated at 31°C, 33°C and 37°C and 5% CO<sub>2</sub> for the remaining days of differentiation culture. A medium change with CnT-Prime 2D and calcium chloride was performed every 2 days in all conditions. For proteomic analysis, medium supernatants from all culture conditions were collected in duplicate before each medium change from day 17 until day 25 of total culture time. Supernatant samples were stored at –80°C until analysis.

#### Sheet formation assay

From day 17 until day 25 of total culture, the keratinocyte sheets were harvested every second day by enzymatically treating with 0.25% (w/v) Dispase II (Thermo Fisher Scientific) at 37°C for 10 min. After flushing the sheets with PBS, the skin grafts were carefully detached from the cell culture flasks and lifted with two tweezers to see whether the biomechanical stability of the sheets was given at the individual days of culture. For further analysis, representative keratinocyte sheet samples with an inner diameter of 6 mm were taken using biopsy punches.

#### Histology and immunohistochemistry characterization of keratinocyte sheets

For qualitative evaluation of tissue organization, keratinocyte sheet samples were fixed with 4% formalin for 1 h at room temperature, transferred in a 2% agarose plug (Carl Roth GmbH, Karlsruhe, Germany) and carefully spread using two tweezers. Stepwise dehydration through a series of graded alcohols was performed, followed by embedding in paraffin (Paraplast; Biosystems Switzerland AG, Muttens, Switzerland) and slicing into 5-μm sections. To assess the tissue composition and architecture of the keratinocyte sheets, hematoxylin and eosin staining was performed. All sections were analyzed by a single operator using an inverted light microscope (Axiovert 40 CFL and Axioplan II; Carl Zeiss AG, Oberkochen, Germany).

In addition, expression profiles of the keratinocyte sheets with regard to early- and late-stage differentiation markers were analyzed using the primary antibodies anti-desmoglein 3 and anti-filaggrin, respectively. Specifically, sections were transferred to target retrieval solution, high pH (K8004; Dako Denmark A/S, Glostrup, Denmark), in a PT Link (PT100/PT101; Dako Denmark A/S) for 20 min at 97°C, performing a three-in-one procedure (i.e., deparaffinization, rehydration and heat-induced epitope retrieval) on the formalin-fixed, paraffin-embedded tissue sections. A standard immunohistochemistry staining protocol was performed on an Autostainer Link 48 instrument (Dako Denmark A/S) for desmoglein 3 (clone 3G133, mouse IgG1, ab14416; Abcam, Cambridge, UK) and filaggrin (mouse IgG, ab17808; Abcam) at a working dilution of 1:100 in antibody diluent (S2022; Dako Denmark A/S) for 20 min at room temperature. The visualization system consisted of the K4001 and K4008 EnVision horseradish peroxidase/3,3'-diaminobenzidine (DAB) system (Dako Denmark A/S), with hematoxylin as counterstain. Tissue formation, including thickness of the keratinocyte sheets and expression intensity of desmoglein 3 and filaggrin, was evaluated using ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, USA). To measure sheet

thickness, three measurements were carried out on each histology slide. The expression intensity of desmoglein 3 and filaggrin, indicated by intensity of the DAB stain, was quantified using the DAB color deconvolution algorithm of ImageJ [11]. The mean  $\pm$  SD sheet thickness as well as DAB intensity of all samples was given for each time point.

#### Proteomics

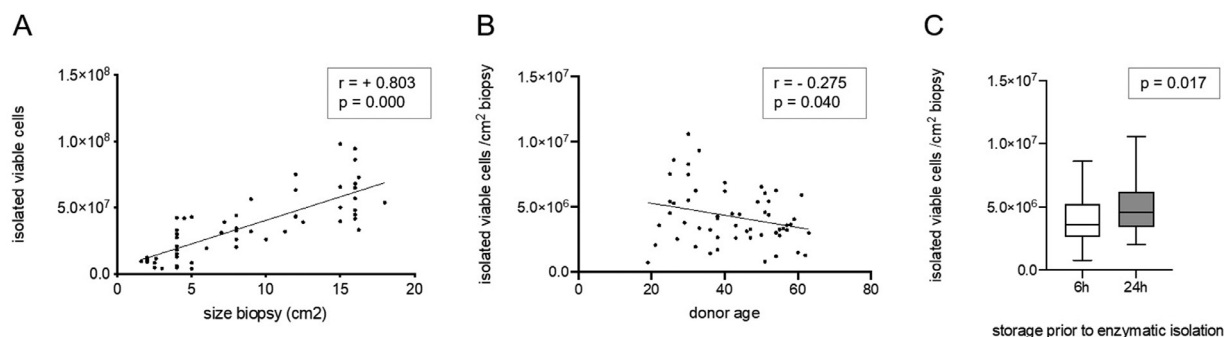
To analyze the metabolic state and intercellular signaling of the keratinocytes within the sheets, targeted microarray using multiplex technology (Luminex; R&D Systems, Minneapolis, MN, USA) was used. With this novel technique, complete profiles of cells and cell cultures can be identified [12]. Magnetic beads coated with the specific antibodies were mixed with the sample (supernatant) and incubated. Thereafter, the bonded analytes were detected using the biotinylated streptavidin complex. Using the Bio-Plex Pro human cytokine 27-plex assay (Bio-Rad, Hercules, CA, USA), 27 parameters, including growth factors/proteins, regulating cell growth and division as well as angiogenesis, were analyzed for each sample. The focus during analysis was on pro- and anti-inflammatory cytokines as well as markers regulating proliferation, differentiation and survival of cells within the keratinocyte sheet. Thus, the following eight analytes were chosen for analysis: interferon gamma (IFN- $\gamma$ ), IL-10, IL-4, IL-1b, IL-8, tumor necrosis factor alpha (TNF- $\alpha$ ), platelet-derived growth factor type BB (PDGF-BB) and vascular endothelial growth factor (VEGF). Data were acquired using a BioPlex 200 reader (Bio-Rad). The mean concentration (pg/mL)  $\pm$  SD of the different secretomes was calculated for each time point and presented over time. For statistical significance, a one-way analysis of variance with a Bonferroni post-hoc test was performed.  $P = 0.05$  indicated the statistically significant threshold.

#### Storability of keratinocyte sheets

To assess the time-dependent usability of the keratinocyte sheets, the storability of the sheets was investigated. Therefore, sheets made of cells from four different patient biopsies (patient age range,  $43.7 \pm 25.2$  years) were cultivated and enzymatically detached as described before. These were then stored either at 4°C in a refrigerator or at 37°C in an incubator (5% CO<sub>2</sub>) in CnT-Prime 2D or CnT-XP3 medium. In addition to temperature, two storage conditions were compared: one with the transport dressing Jelonet (Smith & Nephew, Mississauga, Canada) and one without any dressing ( $n = 4$ ). The viability of the keratinocyte sheets was assessed immediately after harvest (0 h) as well as after 3, 6, 12, 24, 48 and 72 h by a fluorescence-based live-dead assay using fluorescein diacetate (Sigma-Aldrich) and propidium iodide (Sigma-Aldrich). Use of the fluorescent dyes fluorescein diacetate and propidium iodide allowed a simultaneous two-color determination of living cells and the dead cell population, respectively. Keratinocyte sheets of each condition were incubated with 5 mg/mL fluorescein diacetate in acetone and 1 mg/mL propidium iodide in PBS for 5 min. After intensive washing with PBS, the samples were analyzed using an inverted fluorescence microscope as mentioned earlier. The viability of the keratinocyte sheet, indicated by the fluorescence intensity of the fluorescein diacetate stain, was quantified using ImageJ (mean gray value algorithm) and represented as a percentage (mean  $\pm$  SD) in correlation with the sample without storage of the respective medium and temperature over time.

#### Statistical analysis

Statistical analysis was performed using SPSS Statistics 25.0.0.1 (IBM, Armonk, NY, USA). Quantitative data are presented as mean  $\pm$  SD. The individually chosen statistical comparison was specified earlier for each assay.  $P < 0.05$  was considered statistically significant.



**Fig. 2.** Cell isolation efficiency. (A,B) Isolating human keratinocytes from 59 skin biopsies, a possible correlation between biopsy size and isolated viable cells as well as between isolated viable cells/cm<sup>2</sup> biopsy and donor age was investigated. (C) The impact of storage before enzymatic isolation was also analyzed.  $P < 0.05$  was considered statistically significant.

## Results

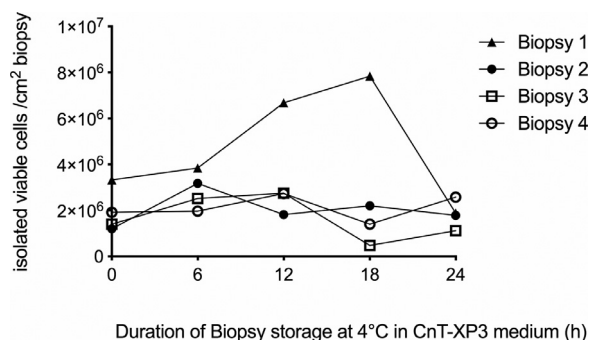
### Cell isolation efficiency

The cell isolation procedure was reliable. Human keratinocytes were successfully isolated from all 59 biopsies, with biopsy surface sizes between 1.6 and 18 cm<sup>2</sup>. The mean number of isolated viable cells was  $4.29 \pm 2.12$  million cells/cm<sup>2</sup> biopsy (Figure 2A). A linear positive correlation between isolated viable cells and biopsy surface area was found ( $r = +0.803$ ,  $P = 0.000$ ) (Figure 2A). A weak negative correlation was found between isolated viable cells/cm<sup>2</sup> biopsy and donor age ( $r = -0.275$ ,  $P = 0.040$ ) (Figure 2B). However, a relation between isolation efficiency per cm<sup>2</sup> biopsy and storage prior to enzymatic isolation was demonstrated (Figure 2C). When comparing cell isolation after 6 h and 24 h of storage, it was shown that the efficiency after 24 h of storage at 4°C was significantly better. The number of viable cells/cm<sup>2</sup> isolated after 6 h of storage was  $3.9 \pm 1.8$  million cells/cm<sup>2</sup>, and this increased after 24 h of storage, reaching  $5.2 \pm 2.1$  million cells/cm<sup>2</sup> ( $P = 0.017$ ). The number of viable cells/cm<sup>2</sup> isolated from split-thickness ( $4.1 \pm 2.8 \times 10^6$  cells) versus full-thickness biopsies ( $4.7 \pm 2.0 \times 10^6$  cells) was not significantly different.

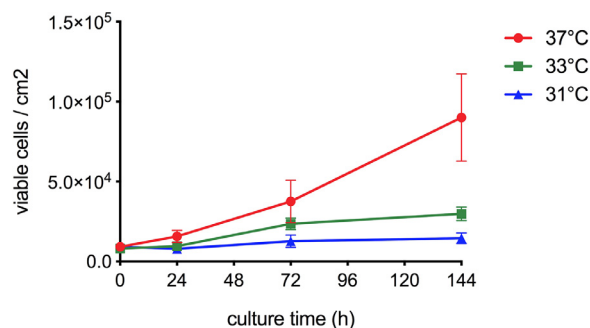
### Biopsy stability

To investigate variability within the samples, biopsies were divided into five equal parts and stored at 4°C in CnT-XP3 maintenance medium until cell isolation. After 0, 6, 12, 18 and 24 h, cells were isolated as previously described.

As can be seen in Figure 3, the number of isolated viable cells/cm<sup>2</sup> biopsy was not significantly adversely affected during the first 12 h of storage. However, after 12 h, some patients showed higher isolation efficiency (see biopsy one), whereas others showed an opposite trend (see biopsy three).



**Fig. 3.** Biopsy storability. Four human skin biopsies were stored at 4°C in CnT-XP3 medium. Over a period of 24 h, cells were isolated every 6 h and the number of isolated viable cells/cm<sup>2</sup> biopsy assessed.



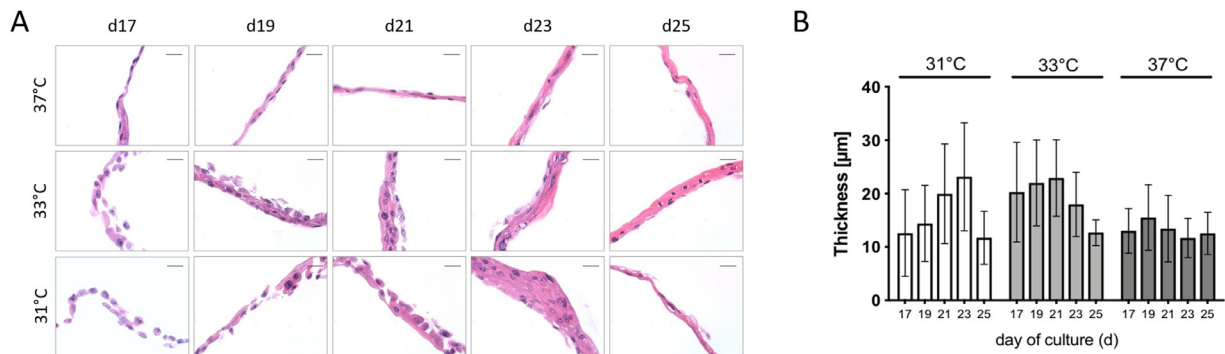
**Fig. 4.** Impact of thermal conditioning on keratinocyte proliferation. Cell expansion was investigated after 0, 24, 72 and 144 h at low and subphysiological temperature of 31°C (blue), intermediate temperature of 33°C (green) and high temperature of 37°C (red). (Color version of figure is available online).

### Thermal conditioning cell expansion

After successful cell isolation, the effect of hypothermic conditioning on the proliferation of human keratinocytes was examined at cultivation temperatures of 31°C, 33°C and 37°C. Thermal conditioning had a significant influence on the expansion behavior of human keratinocytes (Figure 4). Lower temperature conditions of 31°C and 33°C impaired cell expansion compared with 37°C. Population doubling after 144 h at 37°C reached  $3.25 \pm 0.29$  doublings, or 1.7 times that seen at 33°C, with  $1.88 \pm 0.23$  doublings, and five times that seen at 31°C, with  $0.64 \pm 0.25$  doublings. After 144 h, the number of viable cells was highest when cultivated at 37°C ( $9 \pm 2.7 \times 10^4$  cells/cm<sup>2</sup>). This was three times ( $P = 0.011$ ) and six times ( $P = 0.004$ ) higher than that seen in cultures cultivated at 33°C ( $3 \pm 0.4 \times 10^4$  cells/cm<sup>2</sup>) and 31°C ( $1.5 \pm 0.3 \times 10^4$  cells/cm<sup>2</sup>), respectively.

### Keratinocyte sheet formation

The keratinocyte sheets cultivated at all three temperature conditions showed successful sheet formation (Figure 5A). However, thermal conditioning demonstrated a significant influence on the formation of a multi-layer structure. At 17 days of culture, the keratinocyte sheets of the individual patients featured biomechanical stability (i.e., were liftable) when cultivated at 33°C or 37°C. However, the sheets cultivated at 31°C obtained a biomechanical stability at a later time point and were not liftable before day 19. All liftable sheets showed a homogeneous cell multi-layer, and no macroscopic difference between harvesting at day 17 or day 25 was observed. The tissue structure was more compact with cultivation at 37°C compared with that seen at lower temperatures. The thickness of the keratinocyte sheets showed a higher homogeneity during the day 17 to day



**Fig. 5.** Time- and temperature-dependent keratinocyte sheet formation. Keratinocyte sheets cultivated at 31°C, 33°C and 37°C were harvested every second day from day 17 to day 25. (A) Paraffin-embedded and sectioned keratinocyte sheets were analyzed for tissue architecture and sheet formation using hematoxylin and eosin histology staining. (B) Thickness of the keratinocyte sheets was evaluated using ImageJ software (mean  $\pm$  SD,  $n = 6$ ). Scale bar for all panels = 20  $\mu$ m. (Color version of figure is available online).

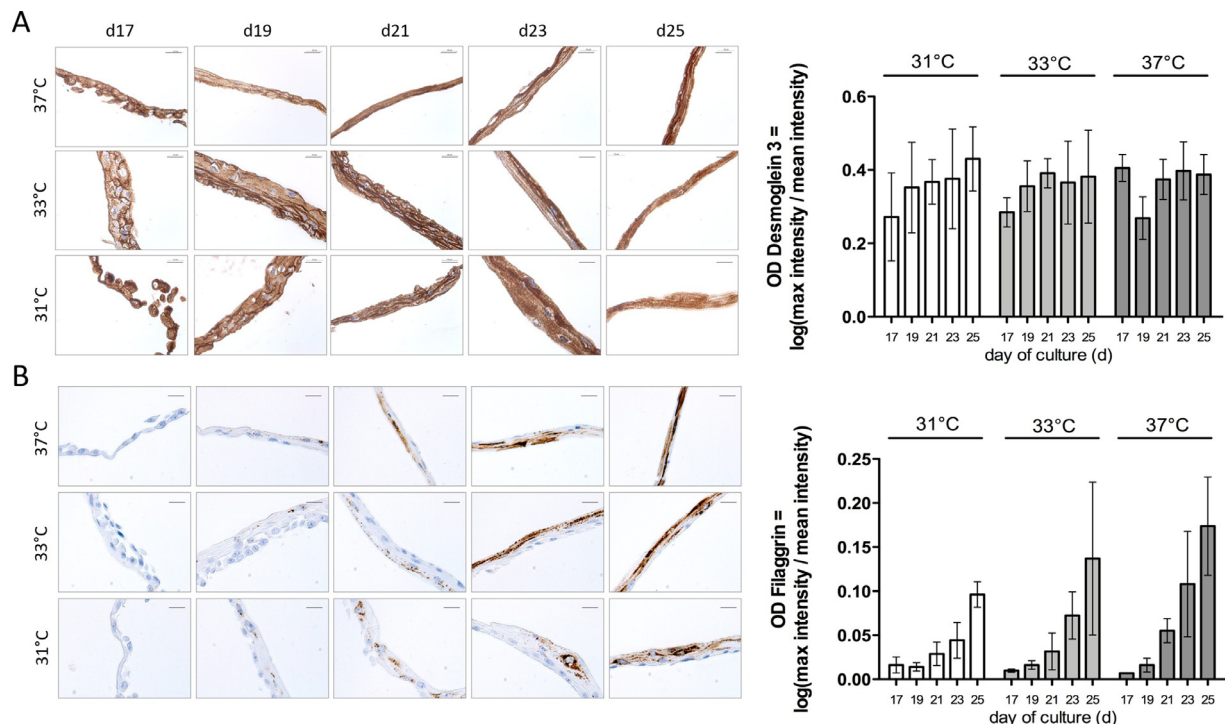
25 window when cultivated at 37°C ( $13.3 \pm 5.0 \mu\text{m}$ ) (Figure 5B). By contrast, sheets cultivated at 33°C were  $18.9 \pm 7.5 \mu\text{m}$  in thickness, and those cultivated at 31°C were even thinner, at  $17.1 \pm 9.1 \mu\text{m}$ . Nevertheless, no statistically significant difference in thickness was detected between the three groups (31°C, 33°C and 37°C).

The early differentiation status of keratinocytes, as indicated by the expression profile of desmoglein 3, did not vary regardless of culture temperature or time (Figure 6A). From the 17th day (earliest time of observation), keratinocytes showed constant expression over time. By contrast, the late-stage differentiation marker filaggrin was increasingly expressed from day 19 onward when cultivated at 37°C and 33°C (Figure 6B). A notably delayed and heterogeneous expression of filaggrin was found at low temperature (31°C).

#### Keratinocyte sheet proteomics

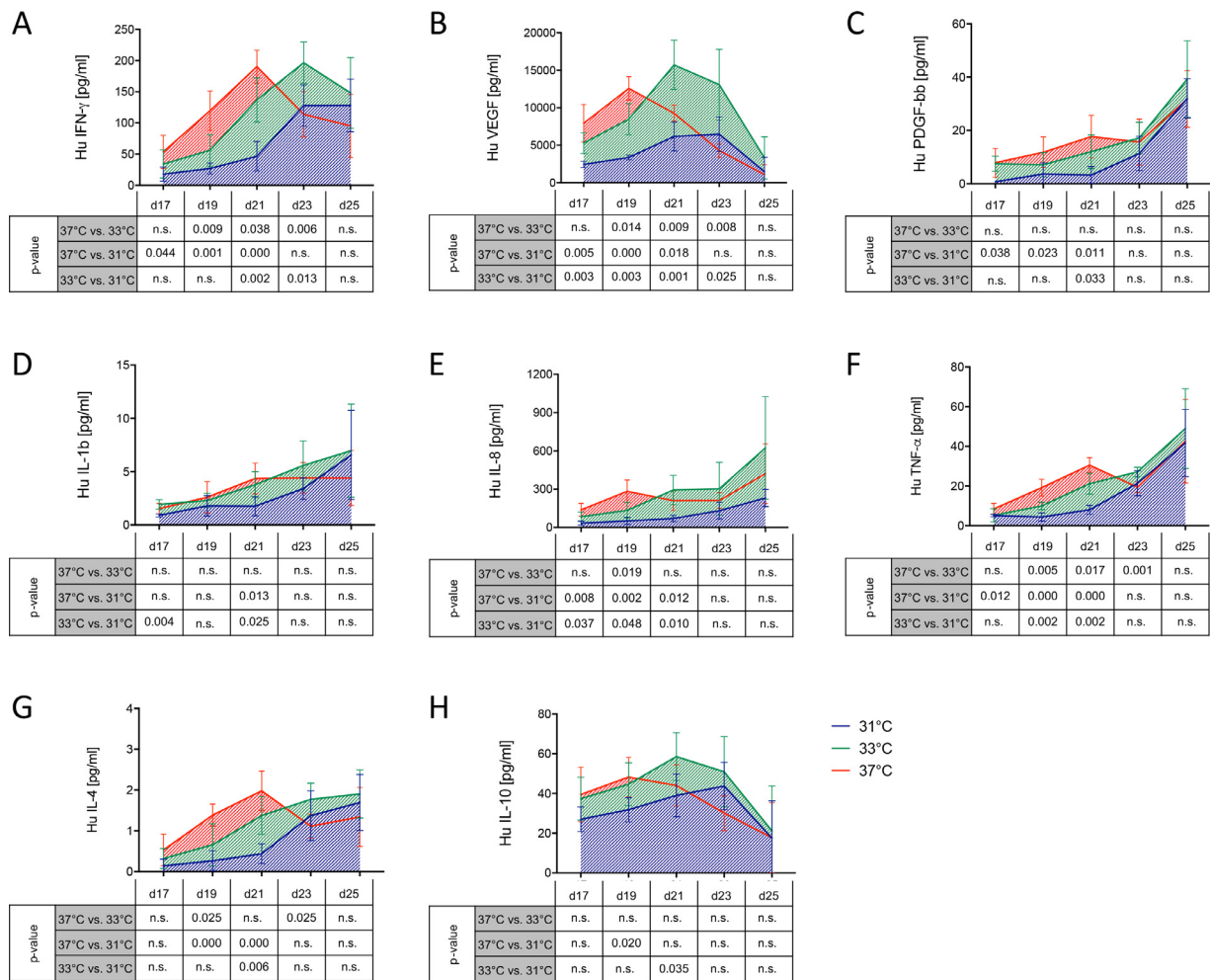
The eight analyzed wound healing-related biofactors (IFN- $\gamma$ , VEGF, PDGF-bb, TNF- $\alpha$ , IL-1b, IL-8, IL-4, IL-10) were detectable in the supernatant of the keratinocyte sheet cultures at all time points and temperatures

tested. Thermal conditioning as well as duration of culture affected the levels of biofactors in both the detected peak and kinetics (Figure 7). Within the sheet harvest window of 17–25 days, those cultured at 37°C exhibited an earlier increase in biofactor release, a narrower peak and a trend toward an earlier decrease in secretion within the time window compared with other temperatures. Sheets cultured at 31°C systematically exhibited lower levels of biofactors and slower release kinetics. With regard to sheets cultured at 33°C, biofactor levels were the highest, and the most sustainable release kinetics occurred in this time window. Specifically, IFN- $\gamma$  release kinetics exhibited a 2-day delay, with levels at 37°C peaking on day 21 ( $190 \pm 26 \text{ pg/mL}$ ) and levels at 33°C rising to approximately the same level ( $197 \pm 33 \text{ pg/mL}$ ) and plateauing at day 23. Levels at 31°C eventually rose starting at day 23. The peak (detected at 37°C and 33°C, extrapolated at 31°C) did not seem to be affected by thermal conditioning, only the kinetics of release. The same profile was observed for TNF- $\alpha$  and IL-4, with levels at 37°C peaking on day 21 (TNF- $\alpha$ ,  $30 \pm 4 \text{ pg/mL}$ , IL-4,  $2 \pm 0.5 \text{ pg/mL}$ ) and levels at 33°C rising to approximately the same level (TNF- $\alpha$ ,  $27 \pm 3 \text{ pg/mL}$ , IL-4,  $1.8 \pm 0.4 \text{ pg/mL}$ ) but continuing to increase. VEGF also showed a temperature-related delay in



**Fig. 6.** Influence of time and temperature on keratinocyte sheet formation. From day 17 to day 25, keratinocyte sheets were immunohistochemically analyzed for graft maturation. (A) Anti-desmoglein 3 indicates the early differentiation phase and (B) anti-filaggrin shows the late-stage differentiation phase of the keratinocytes within the sheets. For quantification of the two stains, ImageJ software was used (mean  $\pm$  SD,  $n = 6$ ). Scale bar for all panels = 20  $\mu$ m. (Color version of figure is available online).





**Fig. 7.** Secretome release of eight analyzed biofactors—the immunomodulatory factor (A) IFN- $\gamma$ ; the angiogenic factors (B) VEGF and (C) PDGF-bb; the pro-inflammatory factors (D) IL-1b, (E) IL-8 and (F) TNF- $\alpha$ ; and the anti-inflammatory factors (G) IL-4 and (H) IL-10—at the three different temperatures, 31°C (blue), 33°C (green) and 37°C (red). Data are shown as mean  $\pm$  SD,  $n = 6$ . Results from the ANOVA post-hoc Bonferroni statistical analysis comparing biofactor levels for each time point are shown in tables under each graph.  $P < 0.05$  was considered statistically significant. ANOVA, analysis of variance; n.s., not significant ( $P \geq 0.05$ ). (Color version of figure is available online).

release, first peaking at 37°C, followed by 33°C and then 31°C. However, the peak levels of VEGF were lowest at 31°C ( $6479 \pm 2337$  pg/mL), followed by 37°C ( $12599 \pm 1570$  pg/mL), and highest at 33°C ( $15730 \pm 3295$  pg/mL), which peaked at day 21 and maintained the highest levels afterward compared with other temperatures. IL-10 showed a similar profile, with less notable temperature-related differences. With regard to PDGF and IL-8, the levels at all temperatures rose progressively over time, with levels at 31°C remaining systematically lower. A progressive increase in IL-1b levels over time was also observed, but the difference between temperatures was less notable.

#### Keratinocyte sheet storability

Overall, keratinocyte sheets harvested at day 21 exhibited a progressive loss in viability when stored at 4°C or 37°C in CnT-2D (growth) or CnT-XP3 (maintenance) medium (Figure 8A,B), maintaining a viability above 50% of the 0 h signal (mean gray value,  $22 \pm 4$ ) for varying amounts of time (Figure 8C–F). A rapid loss in viability with a similar shelf half-life (mean gray value,  $22 \pm 9$ ) was seen in all conditions within the first 12 h of storage regardless of medium or temperature (Figure 8C,E). After 24 h of storage, the viability of the keratinocyte sheets fell below the threshold of 50% of the initial viability signal (0 h) in all conditions. However, after this time point, the loss of cell viability within the keratinocyte sheets slowed down, as indicated by the flattening of the curves (Figure 8C–F). Overall,

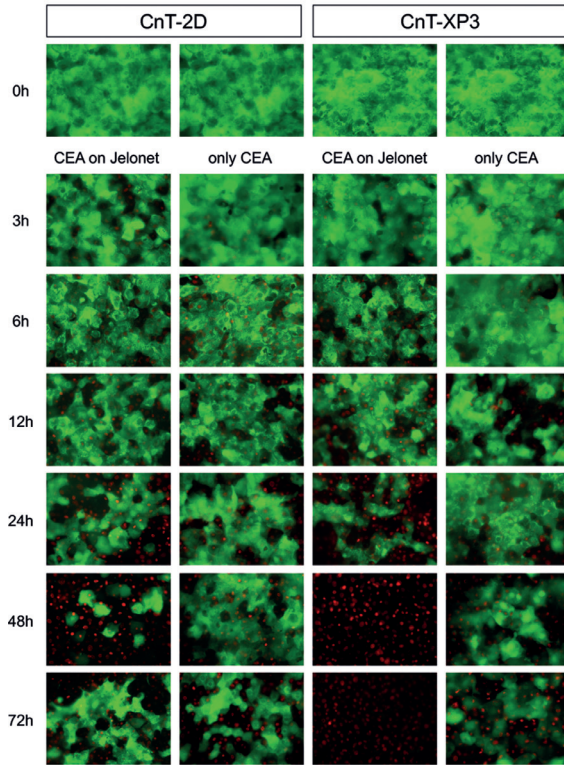
storage at a colder temperature was beneficial regardless of medium or presence of carrier gauze, but this difference was mostly notable from 24 h of storage onward.

In the presence of the transfer gauze Jelonet, cell viability further decreased compared with the control sheet without carrier. A smaller drop in viability due to the carrier was observed when using CnT-2D medium, especially at 4°C. A notable decrease in viability was observed after 48 h of storage in CnT-XP3 medium. Storage on Jelonet at 4°C reduced viability by 25% compared with storage in CnT-XP3 medium only ( $P = 0.015$ ). The same was observed at 37°C, with a 22% decrease compared with the control without gauze carrier ( $P = 0.037$ ). Starting at 6 h, Jelonet reduced viability by 12%. Nevertheless, sheets stored in CnT-2D medium at 4°C on Jelonet maintained viability at  $34 \pm 20\%$  (mean gray value,  $14 \pm 8$ ) for longer storage, up to 72 h (Figure 8C). In the absence of the transfer gauze, a stabilization of viability was detected (Figure 8D,F), with the use of CnT-XP3 medium showing an obvious beneficial effect compared with CnT-2D medium, maintaining the 50% level up to 48 h.

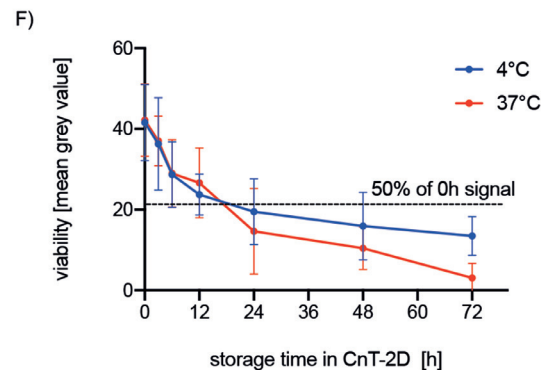
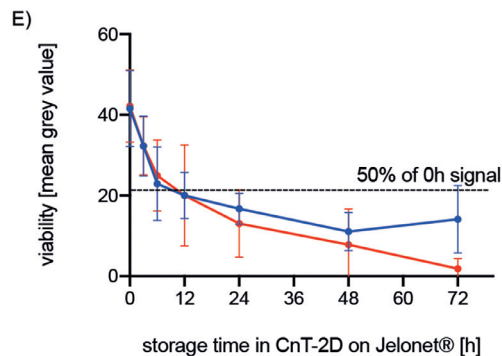
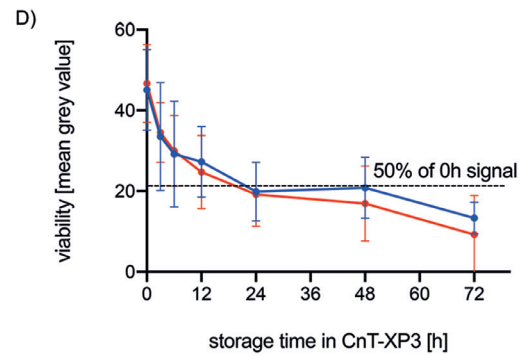
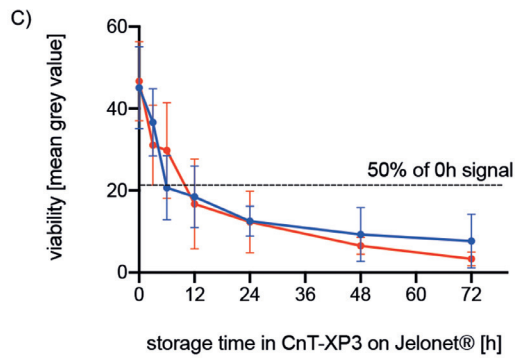
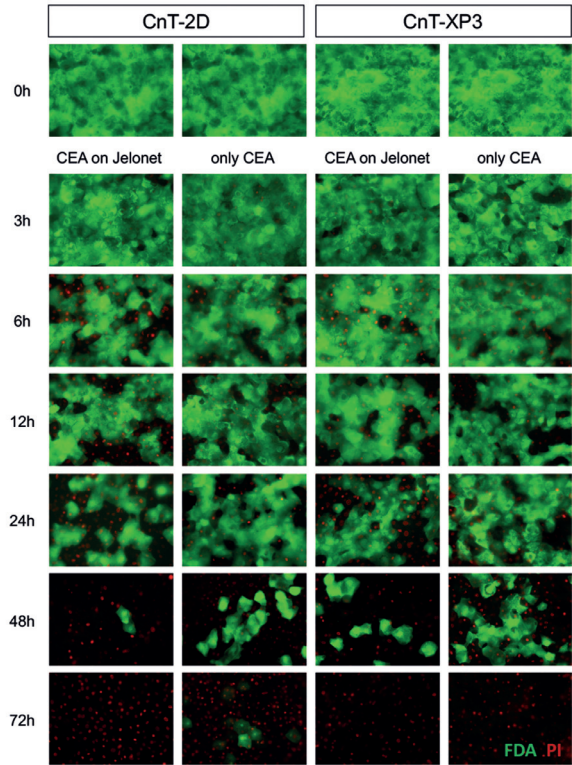
#### Discussion

In this study, the authors demonstrated that temperature has a clear influence on *in vitro* keratinocyte sheet production. Thermal conditioning could be tailored to benefit each manufacturing step. Biopsy cold storage and keratinocyte sheet cold storage were

A) CEA storability at 4°C



B) CEA storability at 37°C



**Fig. 8.** Stability of cultivated keratinocyte sheets. Sheets were harvested and subsequently stored either in CnT-2D or CnT-XP3 medium at 4°C (A) respectively at 37°C (B) on the wound dressing Jelonet® and without wound dressing. After 0, 3, 6, 12, 24, 48 and 72 h, viability was assessed using the live-dead stain fluorescein diacetate/ propidium iodide. To quantify the fluorescent signal of the fluorescein diacetate stain, ImageJ software was used. (C–F) The course of viability over time is represented as the mean gray value of the respective temperature and medium conditions. Mean  $\pm$  SD,  $n = 4$ . (Color version of figure is available online).

beneficial overall. Human keratinocytes showed their maximum proliferation rate at 37°C compared with lower temperatures. During differentiation and sheet formation, keratinocytes benefited from cultivation at 33°C, particularly with regard to improved epidermal structure, biomechanical stability and biological activity (secretome release). Finally, tailored thermal conditioning of the various sheet production steps allowed large-scale sheet production to be ready for implantation by day 18 following biopsy harvest and also allowed for a wider implantability window, from day 18 to day 25, which could then be tailored to the patient's needs.

#### *Production time and hypothermic conditioning of keratinocyte sheets*

Initially, the technique of growing keratinocytes in confluent sheets, with the expansion coefficient needed for large-scale production (i.e., 600 times the surface expansion from biopsy to sheet area), required 5 weeks [13]. Because of intensive process optimization in the last decades, this manufacturing period was subsequently reduced to 3 weeks [14]. Nevertheless, the characterization and harnessing of a transplantation efficiency window would enable surgeons to make an informed decision regarding the timing of a grafting procedure by allowing them to choose the time frame when the patient is most stable and graft maturation is ideal. Determining the duration of the efficiency window would also allow surgeons to work around delays caused by the sudden onset of complications in a patient. This would undoubtedly translate into better clinical outcomes in burn patients in the future.

In this study, the maximal window of transplant efficiency was examined. The aim was to find the earliest time point when keratinocyte sheets can be transplanted despite patient-to-patient variability. To achieve the greatest possible clinical flexibility with regard to transplantation, the extension of the window for a possible transplantation was also examined. The current literature describes degenerative features of cultivated skin transplants in cases of prolonged cultivation (up to 28 days) [8]. After a mature phase of 14–21 days, a senile phase is observable. Therefore, keratinocyte sheet quality was analyzed in the current study between day 17 and day 25 of total production time. With the help of optimized temperature conditions, the individual manufacturing steps should be accelerated and the safety and quality of the sheets optimized.

For this purpose, keratinocytes were isolated from 4 cm<sup>2</sup> human skin biopsies, expanded and cultivated to form multi-layer keratinocyte sheets. Skin tissues were obtained by full- or split-thickness collection from female patients aged 48.5 ± 10.5 years. On average,  $3.2 \times 10^6 \pm 1.35 \times 10^6$  viable epidermal cells/cm<sup>2</sup> tissue were isolated. In accordance with the current literature [15], no differences in cultured keratinocyte doublings were found among cells isolated from the skin of different body areas (breast, abdomen) or from donors of different ages.

To keep the cellular stress level as low as possible when isolating primary cells and thus increase the number of isolated viable cells, the authors examined storage of the skin biopsies in the maintenance medium CnT-XP3. CnT-XP3 regulates tissue homeostasis, thereby reducing cellular stress after biopsy. This observation was confirmed in the present study, showing that storage in CnT-XP3 for 24 h significantly increased the number of viable isolated cells (Figure 2C) compared with isolation without storage. However, a closer look revealed that there were enormous differences in isolation efficiency between individual patients after 12 h of storage. Although in some cases one can benefit from a 4-fold increase in cell isolation efficiency after overnight storage, this is not the case systematically; in fact, the opposite may happen (Figure 3). This patient-to-patient variability, inherent to autologous therapies, means that to reduce overall time until transplantation, the sheet preparation process should be started as soon as possible.

To accelerate keratinocyte proliferation as well as keratinocyte sheet formation, cells were cultivated at temperatures of 37°C and 33°C, which keratinocytes also experience in their native *in vivo* development [10], as well as at subphysiological temperature conditions of 31°C. The comparison confirmed that cultivation temperature *in vitro* has a significant influence on the proliferation of the cells. During the *in vitro* proliferation phase, the keratinocytes benefited from an internal body temperature of 37°C. The two lower temperature conditions showed an adverse effect, with a significantly lower proliferation rate. In other mammalian cell cultures, it has already been demonstrated that a cultivation temperature of 31°C places cells into cold shock and arrests cells in the G1 phase of the cell cycle, which leads to reduced expansion of the cells, decreased biomass and less cells [16]. Since the aim of this study was to accelerate cell proliferation, a keratinocyte expansion temperature of 37°C would be recommended based on these results.

The influence of the three temperatures was also examined with regard to keratinocyte sheet formation between day 17 and day 25 of harvest to assess biomechanical stability, sheet thickness and expression profile of the differentiation markers. Temperature had a profound impact on the differentiation phase of keratinocytes. At 31°C, sheet formation was significantly delayed (day 19), which was reflected in the mechanical stability as well as the expression of the late-stage differentiation marker filaggrin. Overall, the composition of the sheets after cultivation at 31°C was more heterogeneous, which was confirmed by the thickness measurement of the sheets, and biomechanical stability was not reached before day 19 of culture. The subphysiological temperature of 31°C had an obviously negative influence on keratinocyte metabolism with regard to the differentiation capacity of the cells. Keratinocyte sheets cultivated at a mildly hypothermic temperature of 33°C generated better epidermal structures and an increased differentiation potential, which might be associated with increased mitochondrial adenosine triphosphate synthesis [17–19]. Therefore, from a macroscopic and differentiation point of view, forming human keratinocyte sheets at 37°C, and even 33°C, would be preferable to forming them at 31°C. In future studies, it would be valuable to further characterize keratinocyte sheets by looking at stemness markers and adhesion molecules to understand how these may be influenced by thermal conditioning and how they may enhance graft take rate and wound healing capacity.

#### *Proteomics and hypothermic conditioning of keratinocyte sheets*

Upon injury, a sequence of events starts in the epidermis to repair the wound. This healing process is divided into three phases: inflammation, proliferation and maturation. The biochemical mechanisms underlying the wound healing process involve a number of cytokines and growth factors [20]. The generation of a proteolytic environment by inflammatory cells infiltrating the wound site as well as prolonged upregulation of pro-inflammatory cytokines and chemokines inhibits, for example, the normal progression of wound healing. One parameter that influences the productivity and duration of the bio-factor production phase is cultivation temperature. The temperature-dependent release of biofactors has been demonstrated using the kinetics of individual factors [16,21]. With Chinese hamster ovary cells, it has already been shown that the yield of recombinant proteins and their quality can be improved at temperatures below 37°C [22].

As observed with Chinese hamster ovary cells, the experiments carried out in the present study showed that a standard temperature of 37°C led to an early and rapid increase in biomarker secretion in human keratinocytes as well. However, the secretion reached a peak value for only a short time and quickly dropped within the analyzed time window. By contrast, lower temperatures led to longer-lasting secretion of biomarkers, with higher overall yields at 33°C and lower yields at 31°C within the day 17 to day 25 observation window,



which was the graft harvest window. Cultivation conditions of 33°C for keratinocyte sheets resulted in optimal secretion of the angiogenic factors VEGF and PDGF-bb within the observed time window between day 17 and day 25 of culture. The increased level of released angiogenic factors in the keratinocyte sheets could favor the formation of blood vessels after transplantation *in vivo*. Furthermore, dermal wound repair could be improved by the enhanced release of the biomarker PDGF-bb, which is known to be a chemoattractant for fibroblasts, monocytes and neutrophils [23]. Patients have already been treated in the United States with PDGF-bb in completed randomized clinical trials to improve wound healing [24].

The pro-inflammatory biomarkers IL-1 and IL-8 further support the healing process *in vivo* [25]. In addition to the regulation of angiogenesis [26], IL-1 is a highly active and pleiotropic pro-inflammatory cytokine that stimulates keratinocyte growth and collagen synthesis by fibroblasts and regulates hematopoiesis [27] by inducing the production of different hematopoietic growth factors [28]. The release of the biofactor IL-1b seems to be less temperature-sensitive during keratinocyte sheet formation compared with the other biomarkers tested. At all three temperature conditions, a comparable IL-1b release over time was observed. During the inflammatory phase of wound healing *in vivo*, TNF- $\alpha$  is also an important pro-inflammatory mediator. As with pro-inflammatory cytokines, high levels of matrix metalloproteinases have been found in inflammatory states such as chronic wounds. In full-thickness human skin organ cultures, TNF- $\alpha$  stimulates the release of active matrix metalloproteinase 2, which is a type IV collagenase. This suggests a mechanism whereby TNF- $\alpha$  can promote wound healing by indirectly stimulating inflammation and increasing macrophage-produced growth factors [29].

The anti-inflammatory properties of the cytokines IL-10 and IL-4 demonstrate a negative feedback on keratinocytes when cultured at lower temperatures. The ability of IL-10 to facilitate regenerative healing is likely the result of pleiotropic effects through regulation of the inflammatory response [30] as well as upregulation of endothelial progenitor cells. Epidermal keratinocytes are a major source of IL-10 in skin. The production by keratinocytes *in vitro* was most notably upregulated at 33°C compared with the conventional culture at 37°C. The induction of IL-10 may contribute to the anti-inflammatory effect leading to local and systemic immunosuppression after autologous keratinocyte sheet transplantation.

The second investigated anti-inflammatory cytokine, IL-4, is able to activate connective tissue cells and stimulate the accumulation of extracellular matrix macromolecules such as collagen I and III. It regulates extracellular matrix biogenesis via pre-translational mechanisms [31] and might be involved in the regulatory role of epithelial differentiation [32]. In experimental studies, the topical administration of IL-4 to wounds has shown significant acceleration of the healing rate [33]. In the current study, overall higher levels of secreted IFN- $\gamma$  could be observed when sheets were cultivated at 33°C or 37°C. As a multi-functional immunomodulatory cytokine, IL-4 initiates in keratinocytes the host's anti-viral defenses [34]. Furthermore, it is involved in angiogenesis and collagen deposition [35] and induces cytokines specific to mononuclear cells. The IFN- $\gamma$  levels when sheets were cultivated at 31°C seemed to only increase from day 23 onward—a typical delay in secretion shown for many of the investigated biomarkers.

**Table 1**  
Influence of thermal conditioning at 31°C, 33°C and 37°C on secretome release of selected biofactors during keratinocyte sheet formation.

Effect	Analyzed biofactors	31°C	33°C	37°C
Immunomodulatory	IFN- $\gamma$	–	++	++
Anti-inflammatory	IL-10, IL-4	–	++	+
Pro-inflammatory	IL-1b, IL-8, TNF- $\alpha$	–	++	+
Angiogenic	VEGF, PDGF-bb	–	++	+

++ high, + medium, – low release.

These results demonstrate that a cultivation temperature of 33°C beneficially affected the production of immunomodulatory (IFN- $\gamma$ ), anti-inflammatory (IL-10, IL-4) and angiogenic (VEGF, PDGF-bb) biofactors by keratinocytes during sheet formation (Table 1). Pro-inflammatory factors (IL-1b, IL-8, TNF- $\alpha$ ) were also more expressed at 33°C and may play a role in wound healing through a positive feedback loop. Overall, the *in vivo* effect of various biofactors secreted by the cultivated sheets could not be accurately predicted *in vitro*. However, cultivating sheets at 33°C showed the highest and most sustained levels of biofactor release during the investigational graft harvest window 17–25 days after biopsy harvest. This would indicate that such active grafts may exhibit a better take rate and stimulate the wound bed to accelerate wound closure and healing once implanted in patients.

Although this study analyzed the keratinocyte sheet secretome in the supernatant of sheet cultures, it may also be valuable in future studies to determine protein levels in the graft itself and how these may be influenced by thermal conditioning.

### Storability

The regeneration of skin after skin graft transplantation depends mainly on the viability of the graft. Viable grafts facilitate further remodeling of the wound bed *in vivo* compared with non-viable transplants [36]. In case of unforeseen events, the clinical process may be delayed. It is questionable to what extent such short-term storage would affect the viability of enzymatically harvested keratinocyte sheets. It has been shown that cultured epidermal allografts can be stored long-term through cryopreservation [37]. However, to the authors' knowledge, a shelf life between sheet harvest and transplantation has not yet been described. The authors' experiments showed that the first 12 h of storage effected the greatest loss of viability regardless of medium, temperature and presence of transfer gauze. After 24 h of storage, it could be clearly seen that the effect diminished. Compared with the sheets kept in storage without gauze, those placed on Jelonet showed reduced viability. Nevertheless, transfer gauze is necessary for handleability, and therefore investigating various carriers may be valuable for maximizing graft stability prior to implantation.

Although viability drops significantly after 12 h and may reach levels below 50% of initial viability (0 h), the 50% threshold reference is not indicative of clinical efficacy potential. Indeed, sheets may recover after transplantation, and their efficacy in improving wound healing may not require high viability at the time of transplantation. Although there may be indications that the *in vivo* milieu may cause cell death in transplanted cell product [38], the required dosage of viable cells at transplantation has not yet been linked to clinical efficacy. The findings in this study simply show that the tested media had no significant influence on cell viability, but temperature appeared to have an effect, with a lower storage temperature systematically improving viability in all groups at all time points from 24 h onward.

The sheets maintained a similar viability of  $34 \pm 20\%$  (mean gray value,  $14 \pm 5$ ) (Figure 8E) on the transport gauze Jelonet compared with the control without transport gauze (viability,  $32 \pm 11\%$ , mean gray value,  $13 \pm 5$ ) (Figure 8F) when kept at 4°C in CnT-2D medium. When using Jelonet as a carrier gauze, it would therefore be preferable to store harvested sheets at 4°C in CnT-2D medium until transplantation is possible. That being said, investigating alternate carrier materials side by side may be beneficial in improving not only storage viability but also keratinocyte sheet bioactivity and take rate.

### Influence of temperature and pH buffer systems

To maintain the physiological pH value during *in vitro* cultivation, suitable buffer systems have to be used. Thermal conditioning experiments can have the limitation of coupling temperature changes to pH changes simply as a result of CO<sub>2</sub> solubility. The most common buffer

systems include physiological bicarbonate buffer ( $\text{CO}_2$ /bicarbonate) and non-volatile buffers such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Compared with buffering with bicarbonate, HEPES buffer maintains the physiological pH value despite temperature-mediated changes in  $\text{CO}_2$  solubility [39]. At cultivation temperatures below 37°C, HEPES shows more effective buffering for maintaining enzyme structures and functions since its dissociation decreases with decreasing temperatures [40]. In this study, temperature and pH changes could be decoupled using the chosen CELLnTEC medium, which contains both a bicarbonate and a HEPES buffering system. Bicarbonate and HEPES would be resistant to temperature-mediated changes in  $\text{CO}_2$  solubility and therefore the culture medium pH in the temperature ranges used in this study.

### Conclusions

The authors' study may have important clinical implications, in that the expanded window of grafting keratinocyte sheets ( $21 \pm 4$  days after biopsy harvest) will give surgeons the flexibility to decide when a grafting procedure would be most successful based on the patient's health status. Primary keratinocytes display high proteomic consistency related to constitutional factors such as sex, age and anatomic site [41]. However, hypothermic conditioning clearly affects the secretion of biofactors during keratinocyte sheet culture. The optimized temperature conditions of 37°C during the cell proliferation phase and 33°C within the differentiation phase of human keratinocytes reflect the native temperature gradient in normal healthy skin tissue [17]. By using 37°C during the proliferation phase and 33°C during the sheet formation phase of human keratinocytes, the production of cultivated skin grafts can be accelerated and the quality of the grafts, as well as their bioactivity, can be improved, particularly at 33°C. Furthermore, using 4°C during biopsy storage and sheet storage maximizes cell yield and viability. With this protocol optimization, a flexible time window of day 18 to day 25 was created in which a transplantation could take place. These results could be of great benefit with regard to future clinical approaches to the production of high-quality keratinocyte sheets and could significantly improve the treatment of burn patients.

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### Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

### Author Contributions

Conception and design of the study: L. Frese, S.E. Darwiche, M. Calcagni. Acquisition of data: L. Frese. Analysis and interpretation of data: L. Frese, S.E. Darwiche, M. Calcagni. Drafting or revising the manuscript: L. Frese, S.E. Darwiche, B. von Rechenberg, S.P. Hoerstrup, P. Giovanoli, M. Calcagni. All authors have approved the final article.

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